

LC/PDA/ESI-MS Profiling and Radical Scavenging Activity of Anthocyanins in Various Berries

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Anthocyanin extracts of two blueberries, *Vaccinium myrtillus* (bilberry) and *Vaccinium ashei* (rabbiteye blueberry), and of three other berries, *Ribes nigrum* (black currant), *Aronia melanocarpa* (chokeberry), and *Sambucus nigra* (elderberry), were analyzed by high-performance liquid chromatography coupled with photodiode array detection and electrospray ionization - mass spectrometry (LC/PDA/ESI-MS). Both bilberry and rabbiteye blueberry contained 15 identical anthocyanins with different distribution patterns. Black currant, chokeberry, and elderberry contained 6, 4, and 4 kinds of anthocyanins, respectively. The radical scavenging activities of these berry extracts were analyzed by using 2,2-diphenyl-1-picrylhydrazyl (DPPH). All these extracts showed potent antiradical activities.

INTRODUCTION

Anthocyanins (Figure 1) are representative plant pigments widely distributed in colored fruits and flowers. They also exhibit antioxidant activities and therefore may contribute to the prevention of heart disease, cancer, and inflammatory disease [1, 2, 3, 4, 5, 6]. Berries have been known to contain anthocyanin pigments abundantly and thus have been used globally as a medicine or a source of health food/dietary supplement. The amounts and distribution of anthocyanins in the berries differ depending on their plant species, cultivation conditions, and producing districts. Consequently, the antioxidant activity may be different among various berry extracts, in particular, the berry anthocyanin extracts in the commercial market. Although a number of researches for anthocyanin profiling and antioxidant activity of various berries have been reported [7, 8, 9, 10], comprehensive comparison of various berry anthocyanin extracts circulating in the Japanese market is still worth investigation.

In the present study, the extracts of two blueberries, *Vaccinium myrtillus* (bilberry) and *Vaccinium ashei* (rabbiteye blueberry), and three other berries, *Ribes nigrum* (black currant), *Aronia melanocarpa* (chokeberry), and *Sambucus nigra* (elderberry), were analyzed by high-performance liquid chromatography (HPLC) coupled with photodiode array detection and electrospray ionization - mass spectrometry (LC/PDA/ESI-MS). Bilberry is one of a lowbush wild blueberry found in Northern Europe natively. Its extract has been recognized as a medicine

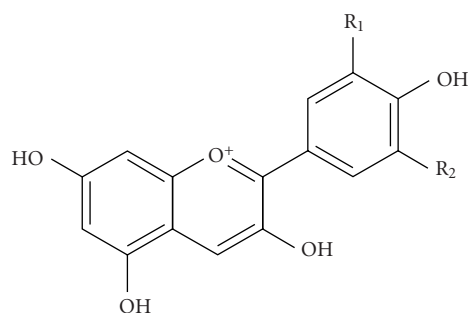


FIGURE 1. Structural formula of anthocyanidins. Cyanidin, $R_1 = \text{OH}$, $R_2 = \text{H}$; delphinidin, $R_1 = R_2 = \text{OH}$; peonidin, $R_1 = \text{OCH}_3$, $R_2 = \text{H}$; petunidin, $R_1 = \text{OCH}_3$, $R_2 = \text{OH}$; malvidin, $R_1 = R_2 = \text{OCH}_3$.

in Italy in the field of ophthalmology [11, 12]. Rabbiteye blueberry is a cultivated species for eating raised in relatively warm regions like Japan. Oral intake of a mixture of black currant anthocyanins, prepared from black currant fruit, has been claimed to be beneficial to visual functions [13]. Elderberry extract was reported to possess some anti-inflammatory activity [14] and antiviral activity against influenza viruses [15]. LC/PDA/ESI-MS, a powerful tool for the analysis of anthocyanins [7, 16, 17], allowed the simultaneous determination of all anthocyanins in plant extracts. We used LC/PDA/ESI-MS for comprehensive profiling of anthocyanins in berry extracts. The radical scavenging activity was also examined in those berry extracts.

MATERIALS AND METHODS

Reagents

Chemicals and HPLC solvents were purchased from Sigma-Aldrich, Wako Pure Chemical Industries, Ltd, or Kanto Kagaku, and were of at least analytical grade. Anthocyanin standards (delphinidin chloride, cyanidin chloride, cyanidin 3-glucoside chloride, peonidin chloride, peonidin 3-glucoside chloride, malvidin chloride, malvidin 3-glucoside chloride, and malvidin 3-galactoside chloride) were purchased from Extrasynthese.

Preparation of extracts

Commercially frozen fruits (300 g) of bilberry, rabbiteye blueberry, and black currant were purchased. The fruits were homogenized in 450 mL of 90% ethanol (0.1% H₂SO₄) and stirred overnight at room temperature. After centrifugation at 3 000 rpm for 5 minutes, the supernatants were filtered and applied to a column of non-ionic polymeric absorbent (Amberlite XAD-7, Rohm and Haas, Philadelphia, Pa) followed by washing with water. Anthocyanin fraction was then collected by elution with aqueous ethanol (0.05% citric acid). Commercial juice concentrates of chokeberry and elderberry were also purchased and applied to the purification steps as above. Purified fractions obtained from the five berries containing anthocyanins were concentrated and freeze-dried to powder.

Total anthocyanidin contents in berry powders

The powders of each berry extract containing anthocyanins dissolved in methanol (2% HCl) were hydrolyzed at 80°C for 30 minutes to obtain corresponding anthocyanidins. Peak values of absorbance around 500–600 nm of each anthocyanidin solution were detected using UV-1700 UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan) to determine the anthocyanidin amounts as delphinidin equivalents.

LC/PDA/ESI-MS analysis of anthocyanins in berry extracts

Powders of each extract were dissolved in H₂O to the concentration of 2 mg/mL followed by filtration with 0.45 μ m nylon membrane and applied to LC/PDA/ESI-MS analysis [17]. The analysis was performed on an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, Calif) using a Capcell Pak C18 UG120 5 mm column (4.6 mm \times 150 mm, Shiseido, Tokyo, Japan) followed by a Finnigan LCQ^{DECA} mass spectrometer with electron spray ionization source (Thermo Electron, San Jose, Calif). HPLC conditions were as follows: solvent A, 0.1%TFA/H₂O; solvent B, 50%CH₃CN/0.1%TFA/50%H₂O; linear gradient, initial percentage of B (15%) to 60 minutes (30%); column temperature, 40°C; flow rate, 0.5 mL/min. Ultraviolet-visible absorption spectra of anthocyanins were detected by a photodiode array detector (PDA) in the range of

250–600 nm. MS parameters were as follows: ionization mode, positive; sheath gas, nitrogen; capillary temperature, 320°C; capillary voltage, 5.0 kV; full scan acquisition, from 50 to 1000 *m/z* at 2 scan/s. Tandem MS analysis was carried out with helium as the collision gas.

Measurement of 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

The method reported by Blois [18] was generally followed. Each berry powder was dissolved and diluted in ethanol at concentrations of 2.0, 1.0, 0.5, 0.2, and 0.1 mg/mL. Fifty μ L of the diluted extracts were added to 1 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) ethanol solution (100 μ M) and left to stand for 5 minutes at room temperature followed by measurement of the absorbance of the resulting solutions at 517 nm using the spectrophotometer described earlier. The DPPH radical scavenging activity obtained by each berry powder was compared with that of Trolox, an analog of vitamin E.

RESULTS AND DISCUSSION

Anthocyanin contents of five berry extracts were analyzed using LC/PDA/ESI-MS. Figure 2 shows a typical chromatogram of the anthocyanins on PDA with selective wavelength (500–550 nm absorption). Extracts of bilberry and rabbiteye blueberry showed 13 identical peaks, although their intensities were different (Figures 2a and 2b). Six, four, and two peaks were obtained from the extracts of black currant, chokeberry, and elderberry, (Figures 2c, 2d, and 2e).

The peaks were also analyzed and identified continuously by mass spectrometry. Anthocyanins of bilberry extract were confirmed to contain five anthocyanin aglycons, that is, delphinidin, cyanidin, petunidin, peonidin and malvidin (Figure 1). Mass chromatograms of each aglycon (Figure 3) indicated that bilberry and rabbiteye blueberry possessed 15 anthocyanins; nevertheless, the extracts showed only 13 peaks on the PDA chromatogram. This is due to the coelution of two different metabolites. Namely, peak numbers 16 and 19 on PDA chromatogram (Figures 2a and 2b) each consisted of two independent anthocyanins, pceonidin 3-glucoside (16a) and malvidin 3-galactoside (16b) on peak 16, and peonidin 3-arabinoside (19a) and malvidin 3-glucoside (19b) on peak 19. A similar result was observed in the case of elderberry extracts. On PDA chromatogram, only two peaks could be detected (Figure 2e, peak numbers 1 and 7). However, mass chromatogram revealed that those peaks each consisted of two independent anthocyanins, cyanidin 3,5-diglucoside (1a) and cyanidin 3-sambubioside-5-glucoside (1b) on peak 1, and cyanidin 3-sambubioside (7) and cyanidin 3-glucoside (8) on peak 7 (Figure 4). One additional large peak was also detected on mass chromatogram of *m/z* = 611 at 58 minutes (Figure 4), although the peak might reflect some flavonoid other than anthocyanin since the compound had no absorbance

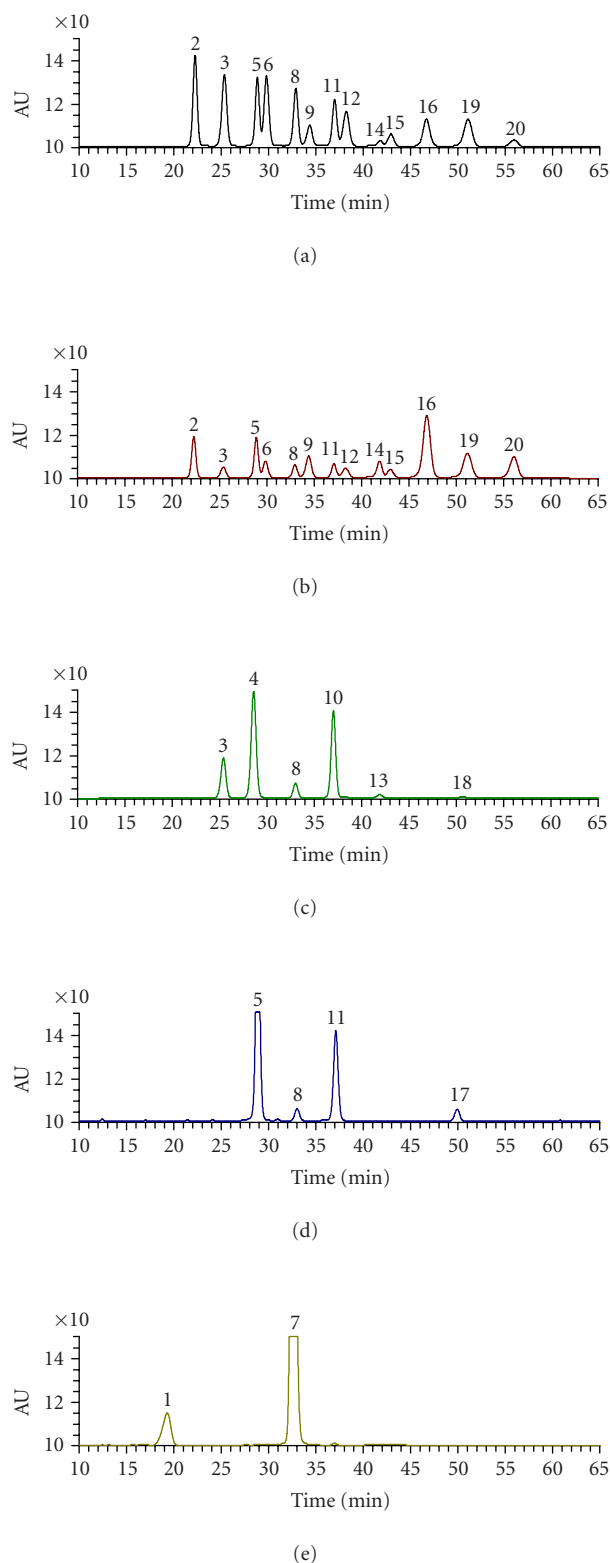


FIGURE 2. HPLC chromatogram of berry extracts on PDA. The chromatograms were obtained on PDA of selective wavelength (500 to 550 nm). Numbers on the chromatograms indicate detected peak numbers corresponding to the numbers in Table 1. (a) Bilberry; (b) Rabiteye; (c) Black currant; (d) Chokeberry; (e) Elderberry.

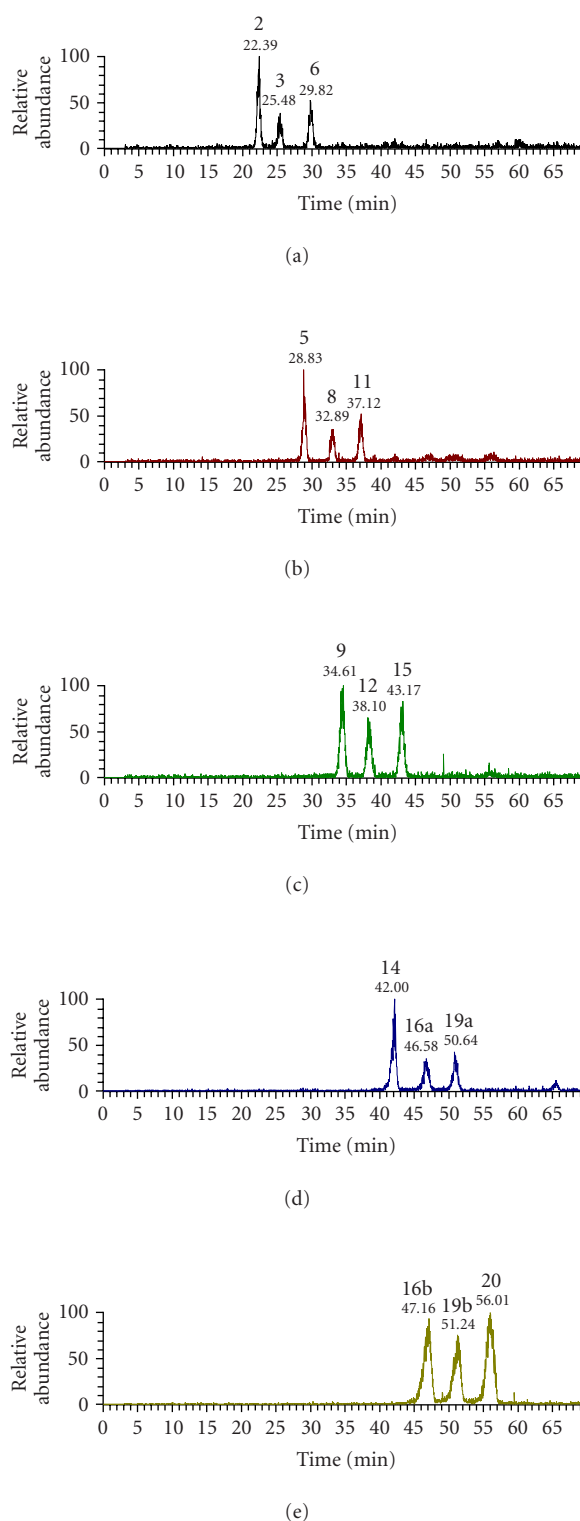


FIGURE 3. Mass chromatogram of bilberry extract selected by m/z of each aglycon. Mass chromatogram revealed that bilberry extract contains 15 anthocyanins; nevertheless, the extract showed only 13 peaks on PDA. The peaks of 16 and 19 in Figure 2 overlap with 16a and 16b, and 19a and 19b, respectively. (a) Delphinidin ($m/z = 303$); (b) cyanidin ($m/z = 287$); (c) petunidin ($m/z = 317$); (d) peonidin ($m/z = 301$); (e) malvidin ($m/z = 331$).

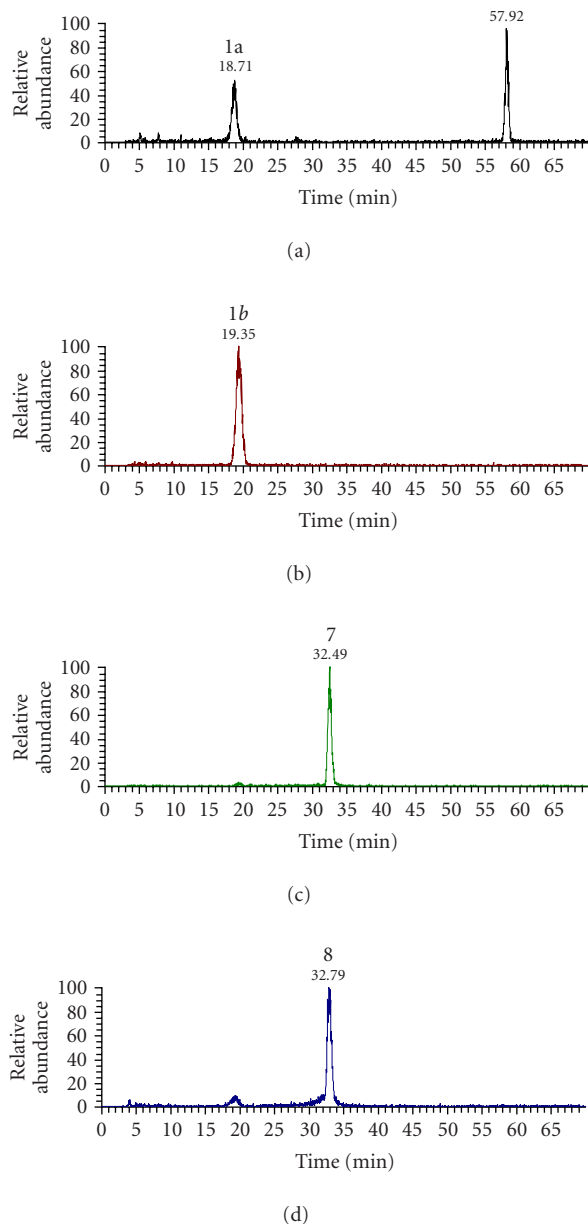


FIGURE 4. Mass chromatogram of elderberry extract selected by m/z of each anthocyanin. Mass chromatogram revealed that elderberry extract contains 4 anthocyanins; nevertheless, the extract showed only 2 peaks on PDA. The peaks of 1 and 7 in Figure 2 overlap with 1a and 1b, and 7 and 8, respectively. (a) Cyanidin 3,5-diglucoside ($m/z = 611$); (b) cyanidin 3-sambubioside-5-glucoside ($m/z = 743$); (c) cyanidin 3-sambubioside ($m/z = 581$); (d) cyanidin 3-glucoside ($m/z = 449$).

at 500–550 nm on PDA (Figure 2). These results implied that in LC-MS analysis, complete separation by HPLC is not necessarily required since mass chromatography can separate compounds accurately.

Total anthocyanidin contents of berry powders prepared in the present study calculated as delphinidin equiv-

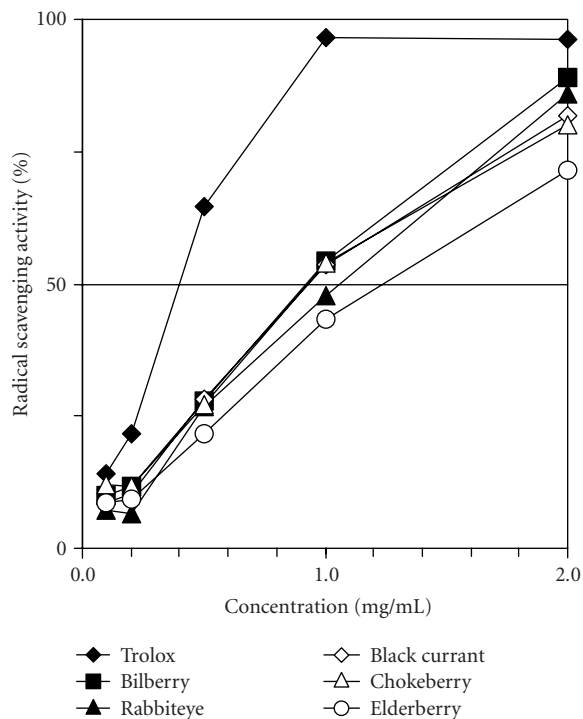


FIGURE 5. Radical scavenging activities of berry extracts. The berry extracts were incubated with DPPH for 5 minutes, and the absorbance at 517 nm due to DPPH radical was determined. Trolox was used as a positive control.

alents per 100 mg of powder after hydrolysis were determined as follows: 28.8 mg (bilberry), 24.8 (rabbiteye), 16.0 (black currant), 19.1 (chokeberry), and 30.4 (elderberry).

Table 1 summarizes the data of LC/PDA/ESI-MS and anthocyanin composition in five berry extracts. Anthocyanins found in bilberry and rabbiteye blueberry consisted of delphinidin, cyanidin, petunidin, peonidin, and malvidin attached with galactose, glucose, or arabinose at the C-3 position [7, 11]. The amount of delphinidin and cyanidin glycosides detected from the extract of bilberry was more than that of rabbiteye blueberry. Delphinidin and cyanidin 3-rutinosides were the main pigments found in black currant [7, 19]. A small amount of petunidin and peonidin 3-rutinosides were detected as well, although they were tentative and remained to be identified. Chokeberry contained only cyanidin as an aglycon, which attached mainly with galactose and arabinose [8, 20]. Plenty of cyanidin 3-sambubioside and cyanidin 3-glucoside were found in elderberry extract [21, 22]. In addition, some of their 5-glucosides were also detected, suggesting that only elderberry possesses a cyanidin 5-glucosyltransferase activity among those berries [23, 24].

Figure 5 shows radical scavenging activity of the berry extracts using DPPH. All five berry extracts exhibited potent radical scavenging activities, though weaker than that of Trolox. They showed relatively similar activities;

TABLE 1. Profiling of anthocyanins in various berries by HPLC/PDA/ESI-MS. Peak intensity (shown as +) was determined from each peak height on PDA chromatogram. Retention time (Rt) on PDA chromatogram. Plus and minus marks stand for the following: (barely detected) +/– < + < ++ < +++ < ++++ < +++++ (abundant).

Peak no	Compound name	Rt (min)	Molecular (m/z)	Fragment (m/z)	Bilberry	Rabbiteye	Black currant	Chokeberry	Elderberry
1a	cyanidin 3,5-diglucoside	19.27	611	449, 287					++
1b	cyanidin 3-sambubioside-5-glucoside		743	449, 287					
2	delphinidin 3-galactoside	22.27	465	303	++++	++			
3	delphinidin 3-glucoside	25.35	465	303	+++	+	++		
4	delphinidin 3-rutinoside	28.51	611	303			++++		
5	cyanidin 3-galactoside	28.83	449	287	+++	++		+++++	
6	delphinidin 3-arabinoside	29.79	435	303	+++	+			
7	cyanidin 3-sambubioside	32.51	581	449, 287					+++++
8	cyanidin 3-glucoside	32.89	449	287	+++	+	+	+	
9	petunidin 3-galactoside	34.33	479	317	++	++			
10	cyanidin 3-rutinoside	36.89	595	287			++++		
11	cyanidin 3-arabinoside	36.99	419	287	+++	+		++++	
12	petunidin 3-glucoside	38.21	479	317	++	+			
13	petunidin 3-rutinoside	41.75	625	317			+/-		
14	peonidin 3-galactoside	41.78	463	301	+	+			
15	petunidin 3-arabinoside	42.93	449	317	+	+			
16a	peonidin 3-glucoside	46.66	463	301	++	+++			
16b	malvidin 3-galactoside		493	331					
17	cyanidin 3-xyloside	49.72	419	287				+	
18	peonidin 3-rutinoside	50.47	609	301			+/-		
19a	peonidin 3-arabinoside	51.07	433	301	++	++			
19b	malvidin 3-glucoside		493	331					
20	malvidin 3-arabinoside	55.91	463	331	+	++			

however, the activity of bilberry was the highest and that of elderberry was slightly low. Extracts of black currant and chokeberry showed nearly identical levels of radical scavenging activity to bilberry extract; nevertheless, total amounts of anthocyanidins in black currant or chokeberry were nearly half those of bilberry, implying that antioxidant activity is not necessarily parallel with the amount of anthocyanidin aglycons. As is generally well known, berries contain a large amount of phenolic compounds that act as antioxidants besides anthocyanins [8, 10, 25, 26]. Since not only anthocyanins but also such phenolic compounds were likely to be extracted from each berry into the powders we prepared in the present study, the radical scavenging activities of berry powders were contributed by both anthocyanins and other phenolics.

There are already a number of reports on the antioxidant activity of berry extracts by several methods such as oxygen radical absorbance capacity (ORAC) [8, 25] or DPPH radical scavenging capacity [7], indicating that bilberry and black currant possess almost equal antioxidant activities. Chokeberry was also shown to possess strong antioxidant activity [8]. Moreover, delphinidin 3-glucoside (found abundantly in bilberry and black currant), delphinidin 3-rutinoside (found only in black currant), and cyanidin 3-glucoside (found abundantly in bilberry and elderberry) were reported to have relatively strong antiradical activity among various anthocyanins [27]. From these reports and our results, extracts of bilberry, black currant, and chokeberry can be regarded as nice candidates for materials of health-beneficial functional foods from the view of the radical scavenging activity.

Since different berries contain unique patterns of anthocyanins, these berries are good resources of the novel genes involved in anthocyanin production. The genes and enzymes responsible for modification and storage are largely unknown [28]. Molecular study on production of berry anthocyanin would identify new genes necessary for the unique pattern of each berry anthocyanin.

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